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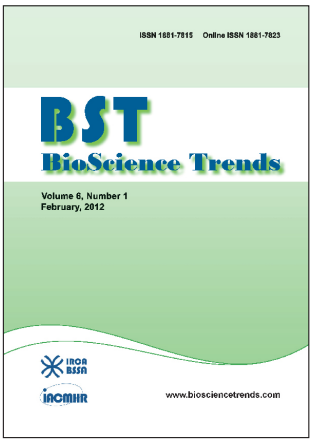

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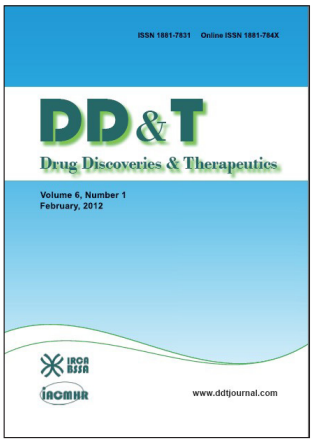

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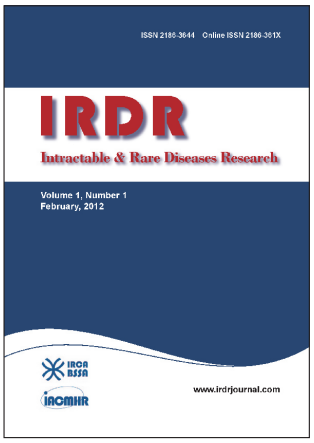

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Editorial and Head Office

Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan

Tel: +81-3-5840-9968, Fax: +81-3-5840-9969
E-mail: office@irdrjournal.com
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Review

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Current situation and prospects of newborn screening and treatment for Phenylketonuria in China – compared with the current situation in the United States, UK and Japan

Lin Mei[1,2], Peipei Song[2], Norihiro Kokudo[2], Lingzhong Xu[1,*], Wei Tang[1,2,*]

[1] *Department of Health Care Management and Maternal and Child Health, Shandong University, Ji'nan, Shandong, China;*
[2] *Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan.*

Summary **Phenylketonuria (PKU) is a treat-able and prevent-able inborn error of metabolism which leads to severe mental retardation and neurobehavioral abnormalities. A screening program, especially for early detection, combined with a Phe-restricted therapeutic diet can help to control the process of PKU of most patients. The China government has put more emphasis on newborn screening and treatment against PKU, yet by comparing the situation of newborn screening and treatment against PKU in China and the relatively developed countries – United States, United Kingdom and Japan, the newborn screening and treatment against PKU in China is relatively weak and many deficiencies are found. More studies concerning multi-stage target blood Phe concentration criteria, a policy that requires newborn screening has to be taken, better financial support for newborn screening, publicity for newborn screening, and national guidelines for treatment of PKU may be prospects in China and may provide some support for better development of newborn screening and treatment against PKU in China.**

Keywords: Phenylketonuria (PKU), newborn screening, treatment, guidelines, policy

1. Introduction

Phenylketonuria (PKU) is an inborn error of metabolism, usually caused by a deficiency of phenylalanine hydroxylase which can lead to mental retardation and neurobehavioral abnormalities (*1*). The overall incidence of PKU in the world varies widely in different human populations such as 1 in 15,000 births in the United States (*2,3*), 1 in 10,000 births in United Kingdom (*4,5*), 1 in 2,600 births in Turkey (*6*) and fewer than 1 in 100,000 births in Japan (*7,8*). However, PKU is a treat-able and prevent-able disease (*9,10*). A screening program, especially early detection, combined with a Phe-restricted therapeutic diet can help to control the development of PKU for most patients (*11-14*).

In China, the overall incidence of PKU is approximately1/11,144 (*15,16)*. Since 1981, a newborn screening program has been implemented by the China government, which mainly focused on congenital hypothyroidism (CH) and PKU. Between 1985 and 2006, according to the accessible data (*17,18*), a total number of 13,666,750 newborns had been tested for PKU and 1,170 cases had been confirmed as PKU patients, with a positive result rate of 1/11,680, and screening numbers for PKU increased remarkably after 1999 (Figure 1). In recent years, the China government also started to put more emphasis on the response to PKU, but since the diagnosis and treatment for PKU in China is still a relatively new area, many deficiencies may exist.

This paper aims to compare the situation of newborn screening and treatment for PKU in China and the relatively developed countries – United States, United

**Address correspondence to:*
Dr. Lingzhong Xu, Department of Health Care Management and Maternal and Child Health, NO.110 mailbox, Shandong University, 44 Wenhuaxi Road, Ji'nan 250012, China.
E-mail: lzxu@sdu.edu.cn

Dr. Wei Tang, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: tang-sur@h.u-tokyo.ac.jp

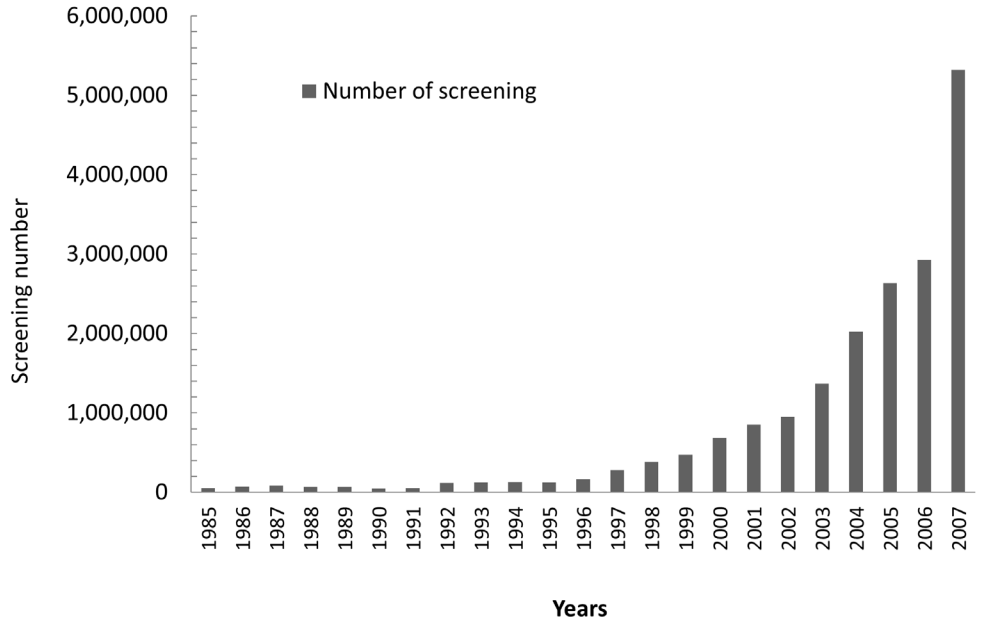

Figure 1. Annual number of newborn screenings in China between 1985 and 2007.

Table 1. The characteristic policy related with newborn screening for PKU in China

Year	Publisher	Policy	Feature
1994	The Central People's Government of the People's Republic of China	Law of Maternal and infant health care	First time identified the importance of newborn screening as a law.
2001	The Central People's Government of the People's Republic of China	The measure for the implementation of the Law of Maternal and infant health care in China	Included the newborn screening into the maternal and infant health care services.
2009	NHFPC	The measure of newborn screening management	Indicated that PKU as one of the screening disease and provided a standardized path for China's newborn screening program.
2009	NHFPC	The plan of newborn screening program	Indicated the objective of newborn screening program, including PKU screening coverage.
2010	NHFPC	The technical specification of newborn screening	Improving the quality of PKU screening and tests.

Kingdom and Japan, and provides some valuable experience for further development of a response policy for PKU in China.

2. Newborn screening for PKU

At present, for almost all patients with PKU, diagnosis from newborn screening and starting treatment immediately leads to a better effect than diagnosis with clinical symptoms (*19*). Newborn screening combined with a Phe-restricted therapeutic diet throughout childhood can help to control the progress of PKU for most patients (*11*). Research shows that with a higher newborn screening rate, earlier treatment can be received by patients, and the prognosis will be better (*12-14*). Therefore, an effective newborn screening for PKU is indeed needed.

A newborn screening program was launched by the China government in 1981, and screening for PKU is a part of the national newborn screening program. The very first screening started in Shanghai in October 1981 (*20*). After that, the screening program in China has made significant progress. The number of screening centers increased from only 3 in the 1980s to 46 in 2002 and further to 179 by the end of 2009 (*18*). The coverage of the newborn screening program also increased from 3.86% in 2003 to 59.01% in 2009 (*18*).

At the policy level, the China government issued a series of policies to enhance the coverage of newborn screenings (Table 1), including identification of the importance of newborn screening as a law. This stipulated that PKU is one of the screening diseases,

Table 2. The policy of newborn screening and treatment for PKU in United States, UK, Japan and China

	Policy of newborn screening against PKU			Policy of treatment against PKU	
	Required by law	Financial support	Publicity supporting	National standardized guidelines	Charging policy
United States	All states required except 4 states: Vermont, Maryland, Minnesota and Idaho.	The test is only free in New York, Pennsylvania, Kansas and District of Columbia. However in other states, health insurance programs and the State Children's Health Insurance Program or Medicaid pay for the fee.	Having some policies and regulations.	Published since 1993	33 states (64.71%) make private insurance requirements about the reimbursement for medical foods for PKU, and 42 states (82.35%) provide state services and assistance for PKU. Only 3 States (Mississippi, Oklahoma and West Virginia) do not have both private insurance requirements and state services and assistance for PKU.
United Kingdom	Do not require yet highly recommend.	The test is covered by National Health Service, and the screening service is free.	Having some policies and regulations.	Published since 1993	The fee of amino acids and low protein foods are fully reimbursed by National Health Service, yet the patients over the age of 16 pay a prescription charge.
Japan	Do not require yet highly recommend.	The test is covered by the government.	Having some policies and regulations.	Published since 1995	The charge for PKU's treatment is covered by national health insurance and special public subsidies.
China	Do not require in most area except Shanghai and Guangzhou.	Free-charge policy in HongKong and Shanghai. Other areas need to charge a fee for screening.	No such specific policy or regulation has been retrieved.	No such specific guideline has been retrieved.	The charge had been covered by URBMI in some places yet no such policy in most regions has been retrieved. Government of Guangzhou covers the fee of PKU children until 8 years of age.

included newborn screening as part of the basic maternal and infant health care services, and issued a standardized path and technical specification for China's newborn screening – in order to improve the quality of PKU screening and testing, and indicated the specific objectives of the newborn screening program (*21-25*).

The newborn screening program for PKU in China has made significant progress since the number of screening centers increased, the coverage of the newborn screening program increased and the number of screened children increased as we mentioned above. However, compared with the newborn screening rate in developed countries, such as 99% in United States, the approximately 60% newborn screening rate in China is still low. To provide a better view of future prospects of newborn screening for PKU in China, we have summarized some potential valuable experience in the United States, UK and Japan.

2.1. *Required by law*

The government enforcement plays an important role in the process of newborn screening for PKU (Table 2). In 1963, in Massachusetts of the United States first passed a law that required the PKU screening test for all infants born in the state and represented the start of the newborn screening program in the United States (*26*). Today, all states of the United States have newborn screening programs for PKU and nearly every newborn is screened shortly after birth (*27*). According to the data, nearly all states required all newborns receive testing by law and only 4 states, Vermont, Maryland, Minnesota and Idaho, indicated that parents can decline screening (*28*). Thanks to these state laws, the launch of the newborn screening program for PKU has legislative authority, as well as improvement of the screening rate in the United States. Participation in the newborn screening program in the United States has reached 99.9% or higher (*29*). For example, Maryland reported that during the last several years, fewer than 5 families opted out of newborn screening each year and in Wyoming, there were 6,800 infants born in 2007, but only 2 families opted out of screening (*29*).

In some areas of China, local government also implemented some specific policies to demonstrate that newborn screening for PKU must be carried out. Shanghai and Guangzhou are two of the earliest areas that carried out newborn screening for PKU in China and also two of the areas with the highest screening rate including 97.0% in Shanghai and

Table 3. Target blood phenylalanine concentrations (μmol/L) as recommended for treatment of PKU in different countries

Age group	United States	UK	Japan	Turkey	China
< 2 years	120 - 360	120 - 360	120 - 240	60 - 240	120 -
2-6 years			120 - 360		
7-9 years		120 - 480	180 - 360		
10-12 years			180 - 480		
13-15 years	120 - 600	120 - 700	180 - 600		
> 16 years	120 - 900		180 - 900		
Incidence	1/15,000	1/10,000	1/70,000	1/4,000	1/11,144

99.0% in Guangzhou (*30,31*). In 1996, the Shanghai government issued regulation of maternal and infant health care in Shanghai which indicated the newborn screening program must be carried out in Shanghai (*32*). While in Guangzhou, regulation of maternal and infant health care was issued in 1998 which formulated that Guangdong province must carry out the newborn screening program and particularly proposed that the screening should include the PKU test (*33*).

Based on the experience in the United States and partly China, government enforcement may be able to help improve newborn screening coverage in China, yet current enforcement is still weak.

2.2. *Multi-stage target blood Phe concentration criterion*

Although the definition of PKU had been determined all round the world, the target blood Phe concentration criterion of PKU still differs significantly among different countries (*11,34-38*) (Table 3).

Based on the information, the United States, UK and Japan have implemented a multi-stage target blood Phe concentration criterion. In Japan, the country with the lowest incidence, has implemented a 6 stage target blood Phe concentration criterion focused on different age groups. For children younger than 2 years old, between 2 and 6 years old, between 7 and 9 years old, between 10 and 12 years old, between 13 and 15 years old, and older than 16 years old, the target blood Phe concentration criterion respectively is 120-240, 120-360, 180-360, 180-480, 180-600, and 180-900. Research shows that after the new treatment guidelines with a more stringent restriction of phenylalanine levels issued in 1995, the mean blood phenylalanine levels of Japanese decreased (*8*). The United States and UK also implemented its own multi-stage target blood Phe concentration criterion based on the national circumstances and received a positive result (*39,40*). In some cases, the exquisite grouping of target blood Phe concentration criteria helps these countries to detect PKU patients more effectively and earlier in order to make a contribution in the direction of PKU control.

According to experience in the United States, UK and Japan, an appropriate multi-stage target blood Phe concentration criterion can help to screen patients more effectively to a certain extent. However, the multi-stage target blood Phe concentration criterion has still not been issued in China. According to the technical specification of newborn screening issued by National Health and Family Planning Commission of the People's Republic of China (NHFPC), the lower target Phe concentration of PKU in China is 120 μmol/L (> 2 mg/dL) and the upper target blood Phe concentration is not specific (*25*). There is no independent lower target Phe concentration for different age groups (*25*).

2.3. *Financial support*

Economic factors play an important role in the process of newborn screening for PKU. In 4 states of the United States, New York, Pennsylvania, Kansas and the District of Columbia, the NBS test is free, while the other states collect a fee. The charge is between $15 (in Florida) and $157.54 (in Rhode Island). Many health insurance programs pay the fees for newborn screening and the State Children's Health Insurance Program or Medicaid can pay the fees for families in need (*41*). Therefore, the vast majority of Americans do not need to pay for the newborn screening for PKU, actually. In Japan, the charge for newborn screening is also covered by the government (*8,42,43*). In UK, the Newborn Blood Spot Screening Programme is part of Public Health England (*44*) covered by the National Health Service, and the screening service is for free (*45*). The same situation has been seen in Japan, the newborn screening for PKU is also covered by the government (Table 2).

The fee for newborn screening for PKU in China is variable in different areas just as in the United States. However, except in Hong Kong, the children in the rest of the areas of China still need to pay a fee (*46*).

2.4. *Publicity supporting*

One of the most important problems during the development of the newborn screening program is publicity – awareness and understanding of newborn screening for the public. In developed countries, the supporting publicity has been implemented for a period and some valuable experience should be reviewed.

In the United States, Federal government support for newborn screening has continued through the years in various ways. Most visible has been the funding

of publicity programs. In 2000, for example, the National Newborn Screening and Genetics Resource Center (NNSGRC), the Health Resources and Services Administration (HRSA) and the Centers for Disease Control (CDC) jointly sponsored a working group meeting to review the issues of implementing tandem mass spectrometry (MS/MS) testing as a means of screening newborns for rare metabolic diseases. The meeting represented the idea that "proposals for planning, operating and evaluating MS/MS for analyzing dried blood spots routinely collected from newborns" and "the public should receive accurate information regarding expanded and comprehensive newborn screening and the evolving knowledge regarding its strengths and weaknesses" (*47*). Combined with the other policies issued in the period (*48,49*), not only awareness towards newborn screening of health practitioners and politicians have been aroused, but also a media awareness campaign during 2000 which led to public attention towards newborn screening (*50*).

The situation in Japan and UK are the same (*51-58*). For example, in Japan, research shows that the awareness ratio of newborn screening in Japan was 26.6% at first, yet after a brief explanatory note on NBS was provided, 71.7% of respondents recognized the necessity of newborn screening (*51*) (Table 2).

In China, there was some research about how to improve the public's awareness of newborn screening (*58-61*) and also some nonprofit websites tried to provide the public with information for newborn screening. However, the situation is still weak.

3. Treatment for PKU

Untreated PKU can lead to mental retardation, seizures, and other serious medical problems (*62*). The acknowledged mainstream treatment for PKU patients is a strict Phe-restricted diet supplemented by a medical formula containing amino acids and other nutrients (*63*). However, the treatment for PKU is variable among different countries.

In China, the idea about early diagnosis and early treatment for PKU had been proposed for a long time (*64,65*), and some reimbursement system has been established (*66-69*). However, there are many issues in the aspect of treatment for PKU in China that still need to be carried out since treatment in China is relatively new. We will expound the situation of treatment for PKU in the aspect of national guidelines and charge policies.

3.1. *National standardized guideline*

In the United States, the Committee on Genetics of the American Academy of Pediatrics and the National Institutes of Health were trying to set up guidelines for PKU since 1993 (*70*), and treatment guidelines against PKU were finally published in 1994 (*71*). In the United States guidelines, the treatment should be initiated no later than 7-10 days after birth and the frequency of monitoring differed by patient's age as weekly until age 1 year, twice monthly from age 1 to 12 years, and monthly after an age of 12 years. As far as treatment duration, treatment throughout life is highly recommended (*71-73*). In UK, the Medical Research Council Working Party on PKU published a series of treatment guidelines for PKU in 1993 (*74,75*). In UK's guideline, the frequency of monitoring is divided into 3 groups as weekly between 0-4 years old, fortnightly between 5-9 years old, and monthly after 9 years old. In Japan, the guideline for the treatment of PKU was revised in 1995 with a more stringent restriction of phenylalanine levels (*8,43*) (Table 2). Although there are some differences between each country's guidelines, guidelines all included the same details as age at start of diet treatment, recommended blood Phe levels, frequency of monitoring, duration of diet treatment and *etc*.

Even though treatment for PKU has been carried out in China for a long time, no national standardized guidelines or regulations about the treatment of PKU issued have been retrieved by us.

3.2. *Charge policy*

In the aspect of charging policy, because of state laws, the situation in different states of America are different – 33 states (64.71%) have private insurance requirements about reimbursement for medical foods for PKU, and 42 states (82.35%) provide state services and assistance for PKU. Only 3 States (Mississippi, Oklahoma and West Virginia) do not have both private insurance requirements and state services and assistance for PKU. Reimbursements focus on patients under 18 to 22 years old, yet Colorado and North Dakota also dictate age limits specially for females 35 years old in Colorado and 45 years old in North Dakota (*76,77*). However, the idea of promoting social support for PKU's treatment has been proposed in the United States since 1993 (*78,79*). In UK, the fee for amino acids and low protein foods are fully reimbursed by the National Health Service, yet patients over the age of 16 pay a prescription charge (*80-82*). In Japan, the charge for PKU's treatment is covered by national health insurance and special public subsidies (*83,84*) (Table 2).

In China, treatment for PKU charged between 15,000 and 25,000 RMB (Chinese Yuan) annually (*85*). This charge had been covered by Urban Residents' Basic Medical Insurance (URBMI) in Fujian province and Hefei city and the reimbursement ratio is more than 70% (*66-68*) yet no such policy in other regions has been retrieved. For rural residents, the China government started a pilot program in January 2013 to cover 70% of the charge of PKU treatment as a

reimbursement for serious illness for less than 10 years old patients with the New Rural Co-operative Medical System (NRCMS) (*69*). Besides these policies, some special bailouts have also been implemented in some regions. For example, in Guangzhou city, children confirmed to have PKU can receive the treatment from the Newborn Screening Center of the City of Guangzhou for free until 8 years of age (*85*).

4. Discussion

4.1. *Prospects for newborn screening for PKU in China*

Although the newborn screening program for PKU in China has made some progress, there are still some challenges among the existing newborn screenings and more effective strategies are expected to be implemented in the future.

The experience of the United States has already showed that appropriate legislation can help to improve screening coverage. Since the policy has been provided its importance during the process of newborn screening for PKU this kind of enforcement has already been implemented in some areas of China and generates an improvement of newborn screening for PKU. Further development about improving China's newborn screening program for PKU, and how to establish the law appropriately needs further discussion and the experience of Shanghai and Guangzhou indicates that the other areas in China can try to implement their own enforcement based on the practical situation.

The multi-stage target blood Phe concentration criteria can not only increase the standardization of the current concentration criteria by subdividing the patients into different age groups, but also can be a symbol of scientific management of PKU. Even though China's PKU incidence is lower than UK's according to the data, the experience of multi-stage target blood Phe concentration criteria that were implemented in these countries can be useful for China to improve the current criteria. However, because the incidence of PKU in the world varies widely in different human populations, research about suitable multi-stage target blood Phe concentration criteria for China still needs to be carried out.

Research also indicates that the important role that economic factors play in the process of newborn screening (*86*), as parents are more willing to let their children be screened when there is less cost for screening. These results remind us that a new charging policy of newborn screening for PKU should focus more on reducing the charge by increasing national financial support or reimbursement in China. The financial support for newborn screening for PKU in the United States, UK and Japan provide a hint of increasing the screening rate. The financial support of newborn screening in China is relatively weak as the majority of families still need to pay for the screening (*87*). The financial support in the United States, UK and Japan is a good example for further study of China's newborn screening policies.

The publicity of newborn screening showed a good impact in the United States, UK and Japan, yet this kind of education in China is still missing. In some areas, researchers try to use health education to introduce newborn screening for PKU to the public, and this kind of research needs to be carried out further in China.

4.2. *Prospects for treatment for PKU in China*

As me mentioned above, the national policy of treatment for PKU in China is still missing, and therefore the standardization of treatment for PKU in China still needs to be enhanced. According to the experience of the United States, UK and Japan, treatment guidelines including details can help to improve the degree of standardization. In the aspect of treatment time, treatment throughout a lifetime can be considered a further aim as lifetime treatment needs the support of quite a lot of health resources. A suitable treatment time for Chinese patients needs more study.

As for charging policy, the current reimbursement ratio of PKU's treatment in China is almost 70% with a 10 years old limitation. Comparing with the situation in United States, UK and Japan, a higher reimbursement ratio and age limitation is a direction for further discussion. However, the local government's attention to reimbursement may be an effective method, such as Guangzhou, HongKong and the situation in different states of the United States.

5. Conclusion

Newborn screening and treatment for PKU in China is relatively weak. Compared with the newborn screening and treatment for PKU in the United States, UK and Japan, the multi-stage target blood Phe concentration criteria, policy that requires newborn screening has to be taken, better financial support for newborn screening, publicity of newborn screening, national guidelines of treatment for PKU including details, and a higher reimbursement ratio and age limitation may be prospects that should be further studied in China and may provide some support for better development of newborn screening and treatment for PKU in China.

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Review

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The molecular and cellular basis of Apert syndrome

Chao Liu[1,2], Yazhou Cui[1], Jing Luan[1], Xiaoyan Zhou[1], Jinxiang Han[1,*]

[1] *Key Laboratory for Rare Disease Research of Shandong Province, Key Laboratory for Biotech Drugs of the Ministry of Health, Shandong Medical Biotechnological Center, Shandong Academy of Medical Sciences, Ji'nan, Shandong, China;*
[2] *School of Medicine and Life Sciences, University of Jinan-Shandong Academy of Medical Science, Ji'nan, Shandong, China.*

Summary **Apert syndrome (AS) is a rare genetic and congenital disease characterized by craniosynostosis and syndactly of hands and feet. AS patients generally require lifelong management, however there are still no effective treatment methods except surgery. In recent years, research has made great progress in the pathogenesis of AS. FGFR2 mediates extracellular signals into cells and the mutations in the *FGFR2* gene cause AS occurrence. Activated FGFs/FGFR2 signaling disrupt the balance of cell proliferation, differentiation and apoptosis *via* its downstream signal pathways. However, how the pathways transform the balance is not well understood and contradictions have occurred in different studies. In this review, we'll focus on these problems to get a better understanding of AS pathogenesis.**

Keywords: Apert syndrome, *FGFR2* gene, pathogenesis, signal pathways

1. Introduction

Apert syndrome (AS) is one of the most severe craniosynostosis syndromes, accounting for about 4.5% of all craniosynostosis, with a prevalence of 1 in 65,000 individuals (*1,2*). AS was first described by Wheaton in 1894 and then reviewed extensively by the French physician Apert (*3*). AS has a dominant inheritance patern, but cases most are sporadic and exhibit a paternal effect. More than 98% of AS cases are caused by *FGFR2* de novo mutations (S252W and P253R) (*4,5*).

Because there are no effective treatment alternatives, many AS patients must have surgery to correct both facial and hand/foot anomalies (*6*). Great progress have been made in understanding the molecular and cellular basis of AS. The disturbance of the FGFs/FGFR2 signal, which changes the balance of proliferation, differentiation and apoptosis of osteoblasts, is ascribed to the development of AS (*7*). Signal pathways which play a vital role in bone formation are also involved in this process (*8*). In this review, we summarize recent studies of AS to have a better and comprehensive understanding of AS pathogenesis.

**Address correspondence to:*
Dr. Jinxiang Han, Key Laboratory for Rare Disease Research of Shandong Province, Key Laboratory for Biotech Drugs of the Ministry of Health, Shandong Medical Biotechnological Center, Shandong Academy of Medical Sciences, Ji'nan, 250062, Shandong, China.
E-mail: samshjx@sina.com

2. AS clinical features

AS also known as acrocephalosyndactyly type 1, though its clinical features are distinctive, acrobrachycephaly is presented in almost all AS cases. In addition, AS affects many other organs and exhibits tissue clinical manifestations including craniofacial, oral, skeletal, cutaneous, respiratory and visceral features.

Based on a clinical study of 136 AS patients, Cohen *et al.* concluded the craniofacial features of AS, including hyperacrobrachycephaly, craniofacial asymmetry, steep wide forehead, flat occiput, downslanting palpebral fissures, divergent upgaze, eso-tropic downgaze, marked depression of the nasal bridge, ocular hepertelorism and proptosis, short and wide nose with a bulbous tip and reduced anterior facial height (*9*).

Oral anomalies are also common conditions for AS patients. Cleft palate or bifid uvula was present in approximately 75% of AS cases (*10*). The intraoral features included impacted teeth, delayed eruption, ectopic eruption, supernumerary teeth and thick gingiva (*11*). An anterior and posterior open bite and crossbite were also observed by occlusal examination (*12*).

AS patients also presented skeletal defects, of which severe syndactyly of hands and feet, which

frequently affected the second, third and fourth fingers or toes, was the most constant. Other skeletal features included decreased glenohumeral mobility, complete glenohumeral ankylosis, short humerus, dramatically short humerus, limited elbow mobility, radiohumeral synostosis, pectus excavatum, flattening of the chest wall, asymmetric chest wall, spina bifida, hemivertebrae, spinal fusions, scoliosis, lordosis, wide interpubic distance, genua valga and osseous ankylosis at the knees (*13*).

Cutaneous manifestations of AS are hyperhidrosis, oily skin, resistant acne, interrupted eyebrows, excessive forehead wrinkling, lateral plantar hyperkeratosis, skin dimpling over joints and oculocutaneous hypopigmentation (*14,15*).

Respiratory complications are presented in about 33% of AS patients with anesthesia complications, which could be severe enough to cause cancellation of surgery (*16*). Based on 12 autopsies of AS patients, reported anomalies of the respiratory system occurred at a lower frequency (1.5%). Of AS visceral features, cardiovascular and genitourinary anomalies are the most common, occurring in 10% and 9.6%, respectively (*17*).

Mental retardation or central nervous system (CNS) anomalies occur in about 55.6% of AS patients and may be partly due to brain malformations or high intracranial pressure (*18*). It also includes megalencephaly, ventriculomegaly, corpus callosum anomalies, hippocampal hypoplasia or dysplasia, hypoplasia or dysplasia of the septum pellucidum, cerebral cortex dysplasia and anomalies of gyral patterning (*19*).

3. The gene mutation of AS

FGFR2 is one of the transmembrane tyrosine kinase receptors FGFRs which are composed of three immunoglobulin-like (Ig) domains in the extracellular region, a transmembrane region and a cytoplasmic tyrosine kinase domain (*20*). It mediates signal transduction from the extracellular into the intracellular area and regulates cell activities through its downstream pathways. FGFR2 is expressed in a wide variety of tissues, for example it is expressed embryonically in early cartilage condensations, proliferating osteoprogenitors, limb mesenchyme, the lungs, brain and skin. Two alternative gene products have been characterized: the IIIb isoform expressed in epithelia and IIIc isoform expressed in mesenchyme and neural tissue. Both isoforms have specific FGF ligands, which control normal development of the organ (*21*).

The *FGFR2* gene is located in 10q26, whose mutations cause AS occurrence. In 1995, it was found that two mutations in the gene: ser252trp of 755C>G and pro253arg of 758C>G in cDNA, which were located in the inner region of IgII and IgIII of FGFR2 (*22*). Patients with the *FGFR2* S252W mutation accounted for about 2/3 of 70 unrelated AS patients, while about 1/3 of AS patients are caused by the P253R mutation, and both of these mutations are the most common mutations of AS (*22*). A study by Oldridge *et al.* demonstrated a new ser252phe mutation in *FGFR2* with CG-to-TT in the cDNA (*23*). In 2002, Kan *et al.* reported the M186T (c.557C>T) mutation of *FGFR2* in AS through genomic screening of fibroblast growth factor receptor 2 (*24*). Next, several rare shear mutations were identified: 940-2A_G, 940-3_-4insAlu and 1041_1042insAlu (*25*). Recently, a novel E731K (c.2191G>A) mutation of exon18 of *FGFR2* in a Korean AS patient was reported (*26*). A brief outline of *FGFR2* mutations in AS patients is presented below (Table 1).

4. The changes of cell activities and signal pathways by mutated *FGFR2*

FGFs/FGFRs signaling plays a vital role in regulating the balance of cell proliferation, differentiation and apoptosis, which is essential to the normal formation of cranial bones. The gain of function mutation of *FGFR2* may disrupt the balance, which may further lead to AS.

4.1. *The effect of mutated FGFR2 on cell proliferation*

FGFR2 has a regulatory role on cell reproduction, whose mutation may impair cell proliferation. In 1998, a study of the calvarial cells from fetuses and infants with AS caused by the *FGFR2* S252W mutation showed normal cell growth (*27*). PCNA immunolocation of the AS fetuses cranial coronal sutures demonstrated no difference between the AS patients and age-

Table 1. The *FGFR2* mutations for Apert syndrome

Disease	Gene	Nucleotide mutation	Amino acid change	Reference
Apert syndrome	*FGFR2*	c.557C>T	p.Met186Thr	*(26)*
		c.755C>G*	p.Ser252Trp	*(22)*
		c.755CG>TT	p.Ser252Phe	*(23)*
		c.758C>G*	p.Pro253Arg	*(22)*
		c.940-2A_G	shear mutation	*(25)*
		c.940-3_-4insAlu	shear mutation	*(25)*
		c.1041_1042insAlu	shear mutation	*(25)*
		c.2191G>A	p.Glu731Lys	*(26)*

* common mutation type.

matched controls (*28*). However, AS skull osteoblasts demonstrated a decreased growth rate compared with normal cells *in vitro* (*29*). Comparative analysis of the proliferation of wild-type (WT) (from 3 individuals) and mutated-type (MT) (from 3 AS patients) fibroblasts and mesenchymal stem cells showed increased cell growth in MT fibroblasts and decreased in MT MSCs (*30*).

AS mouse models with the *FGFR2* S252W or P253R mutation, which represented clinical features of AS patients, provided a good model for the study of the AS mechanism. In 2003, Chen *et al.* introduced the *FGFR2* S252W mutant mouse and analysis of several sutures of different developmental stages E16.5, E18.5, P5 and P8 of the mutant mouse by both BrdU and (^{3}H) thymidine incorporation assays, and demonstrated no significant difference of cell proliferation between mutant and control cells (*31*). Cell proliferation assay of coronal sutures during embryonic calvaria development between E12.5 and E16.5 showed that until E16.5 calvarial cell growth rate of the *FGFR2*$^{S252W/+}$ mouse appeared to be slower than that of WT controls *in vivo*, while compared the P1 calvarial osteoblasts isolated from the *FGFR2*$^{S252W/+}$ mouse and WT littermates *in vitro*, the BrdU incorporation assay showed that the *FGFR2*$^{S252W/+}$ cells have a higher rate of DNA synthesis than the WT controls (*32*). Osteoblasts from limbs of the P0 *FGFR2*$^{S252W/+}$ mouse also proliferated faster than that of normal mice (*33*).

Until now, the influence of mutated *FGFR2* on cell proliferation is controversial, partly due to different subjects and analysis methods applied in different studies.

4.2. *The effect of mutated FGFR2 on differentiation of cells*

Premature fusion of cranial sutures, the main characteristic of AS, may be partly ascribed to the altered differentiation of calvarial cells. Lomri *et al*. analyzed differentiation of calvarial cells by histological analysis, which revealed an increased extent of subperiosteal bone formation and alkaline phosphatase-positive preosteoblastic cells in Apert (S252W) fetal calvaria compared with age-matched controls *in vivo*, and both the expression of alkaline phosphatase and type 1 collagen, and production of the mineralized matrix of Apert cells were higher than controls *in vitro* (*27*). Fragale *et al.* demonstrated that Apert (P253R) calvarial osteoblasts represented higher alkaline phosphatase activity, increased mineralization and expression of noncollagenous matrix proteins than control osteoblasts (*29*). Another study by Lemonnier *et al*. stated that immunohistochemical analysis of the Apert calvaria suture showed higher type 1 collagen, osteocalcin and osteopontin expression in preosteoblasts compared with controls and the expression of the markers in cultured Apert calvaria osteoblasts was also higher than those of normal calvarial osteoblasts (*28*). Long-term cultured human fetal calvarial osteoblasts from two Apert (S252W) fetuses increased expression of osteoblast markers alkaline phosphatase (ALP), type 1 collagen (COLIA1) and osteocalcin (OC) compared to normal osteoblasts (*34*). Tanimoto *et al.* compared differentiation of osteoblasts from the digital bone of two Apert patients with the *FGFR2* S252W mutation and two independent non-syndromic polydactyly patients, and revealed that the Apert osteoblasts showed more prominent ALP, OC and osteopontin mRNA expression and mineralized nodule formation (*35*). In 2012, Yeh *et al.* found both that the coronal suture periosteal fibroblasts and MSCs from three unrelated AS patients showed enhanced osteogenic differentiation compared to cells from age- and sex-matched control subjects *in vivo* and *vitro*(*30*).

Due to the limitation of AS patient samples, animal and cell models of AS have been created. Mansukhani *et al.* compared the differentiation of the mouse calvarial osteoblasts stably transfected with S252W mutated and normal *FGFR2*, and showed that osteoblasts expressing mutated FGFR2 had reduced alkaline phosphatase (ALP) and mineralization (*36*). Another study stated that there was no significant difference in mineralization between chicken calvarial osteoblasts transfected with P253R mutated and WT *FGFR2* (*37*). At the same time, Chen *et al.* also detected no significant difference of expression of osteoblast differentiation markers between *FGFR2*^{S252W} and control mice by *in situ* hybridization (*31*). However, Holmes *et al*. checked osteoblast marker gene expression in E15.5 calvaria, which showed only minor increases in *FGFR2*$^{S252W/+}$ mice compared with WT controls, and ALP activity was higher in *FGFR2*$^{S252W/+}$ osteoblasts in culture than the controls (*32*). A study by Miraoui *et al*. also demonstrated that in mesenchymal C3H10T1/2 cells and calvarial preosteoblast MC3T3-E1 cells stably transfected with WT and S252W mutated *FGFR2* respectively, both cell types expressing MT FGFR2 presented enhanced osteodifferentitation ability by analysis of mineralization, ALP activity and expression of the osteoblast differentiation markers compared with those expressing WT FGFR2 (*38*). A recent study stated primary calvarial osteoblasts derived from *FGFR2IIIc*^{S252W} transgenic mice showed enhanced mineralization, higher ALP activity and greater expression of the differentiation markers than cells from WT mice (*8*).

In most of the above studies, the *FGFR2* mutation which caused the AS enhanced osteoblast differentiation *in vivo* and *vitro*. While up to now, the molecular basis of the altered differentiation of the cell is not well understood. The activation of the signal pathways induced by the mutated FGFR2 may play a vital role in this process.

4.2.1. *ERK1/2 pathway*

The study by Miraoui *et al*. demonstrated that ERK1/2 played a great role in differentiation of C3H10T1/2 cells expressing WT FGFR2 rather than MT cells (*38*). However, in *FGFR2*^{P253R} Apert mouse models, phosphorylation of ERK was increased less than 1.5-fold in the neurocranium compared to WT controls (*39*). In another study activation of ERK1/2 was higher in primary calvarial osteoblasts from the *FGFR2*^{S252W} mouse than normal osteoblasts (*8*). The FGFR2 E731K mutation which was found in a Korean AS patient enhanced phosphorylation and activation of ERK1/2 (*26*). Furthermore, the study by Yin *et al.* revealed inhibition of ERK1/2 activity partly prevented premature closure of coronal sutures (*40*). Another study also verified that the *FGFR2*^{S252W} AS mice treated with U1206, a pharmacological inhibitor of MEK1/2 that blocks phosphorylation and activation of ERK1/2 during pregnancy and early postnatal stages, significantly repressed craniosynostosis and improved skeletal abnormalities. It also showed that a small hairpin RNA targeting the dominant mutant form of FGFR2 (FGFR2S252W) without affecting wild-type mRNA levels completely prevents the Apert-like syndrome in mice, during which the alterations of ERK1/2 activity and FGF-FGFR modulators and downstream genes were observed (*41*). These data clearly demonstrate that ERK1/2 plays a vital role in osteoblast differentiation and AS occurrence.

4.2.2. *AKT pathway*

AKT, a downstream target of phosphphatidylinositol-3-kinase (PI3-K), is an important mediator of cell proliferation and survival *via* phosphorylation of a variety of targets leading to activation or inhibition of their functions (*42-44*). AKT was also reported to promote osteoblast differentiation (*45*). In calvaria tissue and cultured osteoblasts isolated from the *FGFR2*^{S252W} Apert mouse models, Holmes *et al*. detected a significant increase of AKT phosphorylation compared to normal controls (*32*). This indicated that AS osteoblasts enhanced differentiation *via* activation of the AKT pathway. However, in another AS mouse model with the *FGFR2* P253R mutation, phosphorylated AKT was not obviously different compared with that of WT controls (*39*). The different mutations in *FGFR2* may alter osteoblast differentiation *via* different pathways.

4.2.3. *PKC pathway*

Protein kinase C (PKC) is a serine/threonine protein kinase and mediates various cellular functions such as cell proliferation and differentiation (*21*). PKC signaling also regulates osteoblast differentiation and is necessary for bFGF-induced bone formation (*46*). A study by Lemonnier *et al*. demonstrated calvarial osteoblasts isolated from two Apert fetuses with the *FGFR2* S252W mutation represented higher PKC activity than normal osteoblasts. In the following experiments, inhibition of PKC by calphostin C or the PKCα-specific inhibitor, obviously repressed the osteodifferentiation of the mutant osteoblasts (*34*). This may demonstrate PKC pathways are involved in the enhanced differentiation derived from S252W mutated *FGFR2*. In another study, murine mesenchymal C3H10T1/2 cells stably expressing S252W FGFR2 showed enhanced differentiation and increased PKC activity compared with cells expressing WT FGFR2. Pharmacologic inhibition of PKCα slightly reduced matrix calcification in the WT C3H10T1/2 cell, but completely inhibited mineralization induced by MT *FGFR2* (*38*). These experiments confirm that PKC plays a predominant role in mutated *FGFR2* induced differentiation in osteoblasts and mesenchymal stem cells.

4.2.4. *p38 pathway*

The p38 MAPKs belong to the MAPK superfamily and have been shown to be implicated in proliferation, differentiation, apoptosis, senescence and cytokine production (*47,48*). Osteoblasts lacking p38α showed reduced osteodifferentation marker expressions and defective mineralization *in vitro*, which indicated that p38α was an essential positive regulator of osteoblast differentiation (*49*). Holmes *et al.* detected an obvious increase of p38 phosphorylation in the calvarial tissues of *FGFR2*^{S252W} AS mouse models compared to WT controls (*32*). In calvaria tissues from *FGFR2*^{P253R} AS mouse models, Wang *et al.* also detected higher p38 phosphorylation than normal mice (*39*). Another study stated that Apert calvarial osteoblasts showed enhanced differentiation and increased p38 phosphorylation compared to normal cells. Mutant osteoblasts treated with SB203580, a specific p38 inhibitor, significantly inhibited the expression of differentiation markers and obviously reduced mineralization (*8*). These studies show that the p38 pathway plays a vital role in enhanced differentiation of Apert mutant osteoblasts.

4.2.5. *PLCγ pathway*

Phospholipase C (PLC) converts phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) and regulates production of important second messengers which determines cell behavior (*50*). It was reported that sustained Platelet-derived growth factor receptor α signaling in osteoblasts resulted in craniosynostosis by overactivating the Phospholipase C pathway (*51*). Another study stated that human Apert mutant osteoblasts expressed more PLCγ than control cells.

Suzuki *et al.* detected a significant increase of PLCγ phosphorylation in calvarial osteoblasts from AS mouse models with the *FGFR2* S252W mutation compared to osteoblasts expressing sFGFR2IIIc-Ap. However, Apert mutant osteoblasts treated with a PLCγ inhibitor (U73122) didn't obviously reduce its mineralization (*8*). It shows the involvement of the PLC pathways in accelerating osteogenesis in the mutant cells, although it seems to have a weak effect.

4.2.6.*Other signal pathways*

In fibroblasts isolated from AS patients (S252W), JNK phosphorylation is significantly higher than that of the WT osteoblasts. Apert fibroblasts treated with SP600125, a JNK phosphorylation inhibitor reduced its ALP activity and mineralization (*30*). S252W mutated FGFR2 enhanced EGFR and PDGFRα mRNA expression *via* activation of PKCα-dependent AP-1 transcriptional activity. Inhibitation of EGFR and PDGFRα by a specific EGFR inhibitor or the PDGFR inhibitor repressed expression of differentiation markers and reduced mineralization in Apert mutant osteoblasts (*52*). Another study revealed that PDGFR influenced neural crest derived osteogenesis by stimulating the PLC pathway (*51*). These studies confirm that JNK, EGFR and PDGFR pathways are involved in the enhancement of osteoblast differentiation caused by the activated FGFR2.

The mutated *FGFR2* which caused AS drives cells osteodifferentation *via* ERK1/2, AKT, PKC, p38, PLCγ, JNK, EGFR, PDGFR and other signal pathways. Inhibitors specific to the pathways may be helpful for the treatment of AS patients.

4.3. *The effect of mutated FGFR2 on cell apoptosis*

Apoptosis, also called programmed cell death, is a widespread phenomenon which plays a crucial role in a variety of physiological and pathological processes (*53*). A study also indicated that apoptosis was involved in normal and pathological osteogenesis (*53*). In 2000, Mansukhani *et al.* first reported S252W mutated *FGFR2* induced apoptosis in mouse calvarial osteoblasts (*36*). Next, Lemonnier *et al.* also detected significantly increased apoptosis in Apert coronal sutures *in vivo* and cultured Apert calvarial osteoblasts *in vitro*. In addition, they found increased apoptosis induced by S252W mutated *FGFR2* was PKC-dependent *via* overexpression and activation of its downstream signals IL-1 and Fas (*54*). Chen *et al.* demonstrated that the MT FGFR2 increased Bax expression and apoptosis of osteogenic cells in the mutant coronal suture of the $FGFR2^{S252W}$ AS mouse models (*31*). In AS mouse models with the same type mutation in *FGFR2*, it was reported that apoptosis accompanied fusion, but was restricted to bone fronts in contact with one another, while no apoptosis was detected in WT mouse sutures (*32*). However, in another Apert mouse model with P253R mutated *FGFR2*, there was no obvious difference in apoptosis in the coronal sutures between the MT and WT mouse models (*39*). A recent study demonstrated only the inter-premaxillary suture exhibited significantly increased apoptosis in bone bordering the suture mesenchyme in AS mouse models with S252W mutated *FGFR2* compared with normal mice (*55*).

Based on the above studies, we conclude that mutated *FGFR2* induces apoptosis, which has been confirmed by analysis of human bone samples and mouse models *in vivo* and *vitro*. At the same time, we face another question "Whether enhanced apoptosis leads to premature suture closure or is secondary to the craniosynostosis". Chen *et al.* suggested that accelerated cell death possibly reduced the space between osteogenic fronts of flat bones and resulted in physical contact of these bones (*31*). On the other hand, Holmes *et al.* thought apoptosis appeared to be a consequence rather than a cause of sutural fusion based on their study which showed craniosynostosis was an early onset during embryo development while E16.5 apoptosis began to appear in the $FGFR2^{S252W}$ coronal sutures and were strictly limited to sites of osteoid contact between the frontal and parietal bones (*32*). The answer to this question needs more experiments in the future to confirm.

4.4. *Other cell activities altered by mutated FGFR2*

It was reported that mutated *FGFR2* increased cell-cell aggregation and N- and E-cadherin expression in human calvarial osteoblasts. Neutralizing anti–N-cadherin antibody or N-cadherin antisense oligonucleotides suppressed increased cell-cell aggregation and reduced osteoblast differentiation markers overexpression in mutant osteoblasts (*34*). A study by Holmes *et al*. suggested that the critical event of Apert craniosynostosis was to increase the recruitment or advancement of osteoprogenitor cells at the sites where sutures should normally form (*32*). Both of the above studies suggested that cell adhesion and recruitment play important roles in the pathogenesis of AS.

5. The altered chondrogenesis by mutated *FGFR2*

Bone formation is formed through intramembranous and endochondral bone formation. Two studies detected FGFR2 expression in a chondrocyte lineage, which suggested FGFR2 may play an important role in development of chondrocytes (*56,57*). Wang *et al.* found ectopic cartilage at the midline sagittal suture, and cartilage abnormalities in the basicranium, nasal turbinates and trachea in $FGFR2^{S252W}$ mice (*58*). It

indicated that altered chondrogenesis was involved in the occurrence of AS. In another study, chondrocytes with S252W mutated *FGFR2* in hydrogel culture also exhibited strong staining of the cartilage specific marker: collagen type II, while only minimal staining was seen in the WT control (*33*). In another Apert mouse model with P253R mutated *FGFR2*, it presented shortened synchondroses, short trabecular bones and a delayed secondary ossification center in the tibia; which stated that the *FGFR2* P253R mutation in mice resulted in retarded endochondral ossification (*40*). Nagata *et al.* also confirmed that P253R mutated *FGFR2* accelerated maturation and hypertrophy of cranial base chondrocytes, which resulted in disturbance of the cranial base growth with precocious endochondral ossification in mice with the mutation (*59*). Based on the studies, we can conclude that altered chondrogenesis caused by activated *FGFR2* mutation plays a vital role in the occurrence of AS.

6. Acne in AS

Acne is a chronic inflammatory disease and also a clinical manifestation of AS (*60*). Major elements contributed to the acne pathogenesis include abnormal follicular differentiation with hyperproliferation, increased sebaceous gland activity with increased sebum, as well as increased bacterial colonization, inflammation and immunological mechanisms. Androgens play an important role in the stimulation and growth of sebocytes, sebum production, and keratinocyte proliferation in the ductus seboglandularis and the acroinfundibulum (*61,62*). Studies also demonstrated that FGFR2 plays an essential role in homeostasis of the epidermis and sebaceous gland development (*63-65*). FGFR2 generates two splice variants by alternative splicing: FGFR2b and FGFR2c which are expressed in epithelial and mesenchymal cells respectivly. The *FGFR2* mutation in AS altered FGFR2b-mediated downstream signal pathways is involved in pathogenesis of AS (*66*). The mutated FGFR2b altered cell proliferation and MMP expression *via* the MAPK pathway, induced lipogenesis and terminal sebocyte differentiation *via* the PI3K/AKT and Shh/MC5R pathways and induced IL-1a, and inflammatory reactions *via* the phospholipase Cγ/protein kinase C pathway (*66*). Melnik stated that known anti-acne agents which attenuated FGFR2-signaling pathways was a common mode of action (*67*). So, the above studies demonstrated that increased fibroblast growth factor receptor 2 (FGFR2)-signaling caused by the mutated *FGFR2* contributed to the occurrence of acne in AS.

7. Conclusion

After decades of investigation, the changes of proliferation, differentiation and apoptosis in cells have been shown to play a prominent role in the occurrence and development of AS. One purpose of this review is to demonstrate that the pathways regulating the cell behavior changes caused by the *FGFR2* mutation in AS patients has been established (Figure 1). Though these pathways are mostly incomplete, they provide a basis for future advances. *In vivo* and *vitro* studies have shown that these pathways play a vital role in AS and pharmacological inhibitors that specificly repress the activation of these pathways could significantly improve the AS clinical manifestation. The mutated FGFR2b pathway has further lead to the acne occurence in AS. Notwithstanding our extensive knowledge of cell behavior changes by the *FGFR2* mutation through these pathways, different types and differentiation stages of cells display distinct responses to the activated *FGFR2* mutation in AS, which needs further investigation in the future.

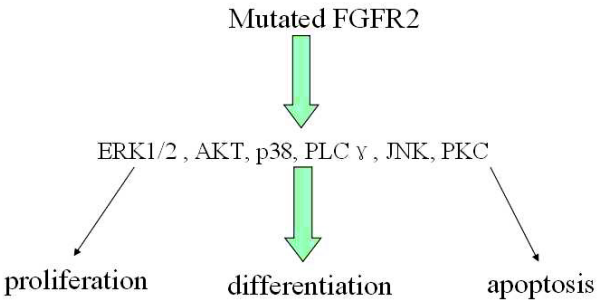

Figure 1. Altered pathways in AS. The mutated FGFR2 which caused AS disrupts the cell balance of proliferation, differentiation and apoptosis of *via* changing the activation of its downstream signal pathways: ERK1/2, AKT, P38, PLCγ, JNK and PKC.

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Case Report

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Prader-willi syndrome: A case report and a Chinese literature review

Junzhen Zhu[1], Qinying Cao[2], Ning Zhang[3], Lijuan Zhao[2]

[1] *The Center of Tri-excellent, The People's Hospital of Hebei Province, Shijiazhuang, China;*
[2] *The Fourth Hospitals of Shijiazhuang, Shijiazhuang, China;*
[3] *The Second Hospital of Hebei Medical University, Shijiazhuang, China.*

Summary **Prader-Willi syndrome (PWS) is a genetic disorder, resulting from lack of gene expression on the paternally inherited chromosome 15. It is important to determine diagnostic methods for PWS for early treatment. In this study, we report a newborn with Prader-willi syndrome. We further summarized the genetic testing results in the Chinese literature and the relevance of high resolution chromosome and genome-wide copy number variation analysis. There is a heterozygosis deletion of a 5 Mb region in the paternal chromosome 15q11.3-q13.3 by genome-wide copy number variation analysis. However, there is no abnormality in high resolution chromosome karyotype analysis. In conclusion, genome-wide copy number variation analysis is an effective and specific diagnosis method, which will provide scientific evidence for the clinical diagnosis and early treatment of PWS.**

Keywords: Prader-Willi syndrome, genome-wide copy number variation analysis, high resolution chromosome gene imprinting

1. Introduction

Prader-Willi syndrome (PWS) is a complex genetic disorder, characterized by neonatal hypotonia, delayed development, short stature, childhood obesity, hypogonadism, characteristic facial features, and so on (*1*). It is a genomic imprinting disorder caused by a deficiency of paternally expressed gene or genes in chromosome 15 (15q11.3-q13.3 region) (*2*). This region contains genes that are epigenetically imprinted. A recent foreign study has shown that the prevalence of PWS is 1/29,000 in newborns (*3*).

Although an accurate consensus of clinical diagnostic criteria exist, it is difficult to make the diagnosis on many patients who are too young to manifest sufficient features, particularly at an early age. The clinical symptoms are difficult to diagnose in infants and only become clearer at later ages as the patients develop hyperphagia and morbid obesity. Therefore, further genetic testing is important to confirm the diagnosis of PWS for all patients. Cytogenetic and molecular genetic diagnosis can confirm PWS at an early stage and provide useful information for the genetic counseling of PWS families. In our study, the child was observed from the prenatal motherhood. We found that there is weak quickening, fewer fetal movements at 39 weeks and intrauterine distress during the pregnancy. The male child was born by cesarean delivery and studied by cytogenetics and genome-wide copy number variation analysis.

**Address correspondence to:*
Dr. Junzhen Zhu, The Center of Tri-excellent, The People's Hospital of Hebei Province, Shijiazhuang 050011, China.
E-mail: zhujzhebei@163.com

2. Case report

The Characteristics of the Object. The male neonatal was clinically suspected to be a PWS patient. This patient is the first child of his parents. There was weak quickening, fewer fetal movements at 39 weeks and intrauterine distress during the pregnancy. Therefore he was born by cesarean delivery and his birth weight was only 2.8 kg. Furthermore, his Apgar score was 3.

Genome-wide copy number variation analysis. Informed consent was obtained from the parents of the patient. The peripheral blood of the patient and his parents were taken for normal chromosome and high

resolution chromosome G banding (*4*). Meanwhile, the blood genomic DNA was isolated by the standard phenol/chloroform method and then tested by genome-wide copy number variation analysis using the Illumina HumanCyto SNP-12 Beadchip.

This patient has low muscle tone, little limb activity, low crying and crying like a cat call syndrome, poor sucking force, feeding difficulties and a characteristic face, which are basically according to the clinical diagnosis standards. The characteristics including white skin color, narrow face, small jaw, almond eyes, small mouth, thin upper lip and convex, angle downward, low ear, slightly larger head, small hands and feet compared to the standard, short torso, lack of wrist radian in both ulnar sides of upper limbs, limited outreach, reproductive organs dysplasia, bilateral cryptorchidism, slightly thin limbs and flat belly. However, the intelligence and appearance of his parents are normal and there are no relatives with similar manifestations compared to the patient.

In this study, we found that there was a deletion of a 5 Mb region in chromosome 15q11.3-q13.3 in this patient by genome-wide copy number variation analysis (Figure 1). Furthermore, it was a regional deletion in paternal chromosome 15q11-q13 in this PWS patient, but there were no abnormalities in conventional karyotype and high resolution chromosome analysis.

Through clinical follow-up, after 1 to 3 months, the child had obvious symptoms, including feeding difficulties, poor sucking, poor physical activity, low and weak cry, and drowsiness. After 6-8 months, appetite was improved. Weight was increasing rapidly, but growth in height was slow. At the age of 1, the child had characteristics of the typical face such as narrow forehead, and almond eyes. Weight was gained rapidly, and intelligence quality was low.

3. Discussion

FPWS is a hereditary disease associated with genomic imprinting. The several mechanisms of PWS include: *i*) a paternally derived large deletion of 15q11-q13, accounting for 70% of all patients; *ii*) a maternal uniparental disomy, accounting for approximately 20%-25%; *iii*) a defect in the genomic imprinting mechanism, about 2%-4%; and *iv*) other rare reasons, such as chromosome translocation and microdeletion, less than 1%.

PWS is considered as the most common cause of life-threatening obesity. As a genetic syndrome, there is no effective treatment method for PWS. In the clinic, we should pay attention if the baby has unexplained low muscle tone, weak sucking force, feeding difficulties, and delayed puberty with moderate mental retardation (*5*). The diagnostic basis for this disease is genetic techniques, which provide an important guiding significance for genetic disease screening and prenatal diagnosis. Therefore, the suspected newborn should have early gene analysis in order to avoid misdiagnosis. In addition, PWS cases do have a significant recurrence risk not only for the relevant parent, but also for certain close relatives, thus it is important to determine the exact molecular defect in addition to the general diagnosis of PWS.

Prenatal diagnosis of PWS is difficult. For the doctor it is very difficult to assess the risk of PWS occurrence during pregnancy for the delivery of PWS child couples (*6*). In order for better genetic counseling, we should carry out ultrasound images to analyze fetal movements during pregnancy. In this study, the activity of the child was weak in his mother's womb before birth. We should further take villi or amniotic fluid cells for molecular diagnosis.

Molecular genetic tests are able to definitively diagnose PWS and allow early diagnosis of the

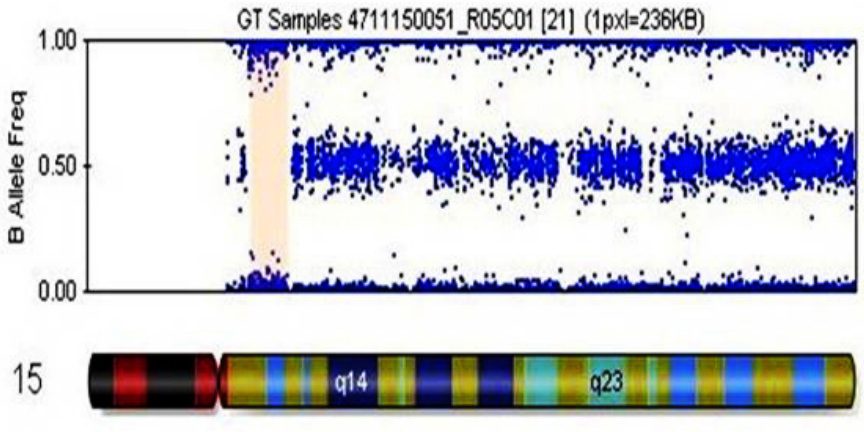

Figure 1. This figure shows a schematic diagram of chromosome 5. Red areas shown are the deletion region (2120838726210229), of 5 Mb, located in 15q11.3-13.3.

Table 1. The Method of Molecular Genetic Tests Reported in China

Methods	Number of reports	Location	Result of testing
Bisulphate sequencing	1	chromosome 15q11.2	Abnormal methylation of the CpG islands
Fluorescence in situ hybridization (FISH)	1	chromosome 15q11.2	Small deletion
Methylation-specific PCR (MSPCR)	9	chromosome 15q11-13	Small deletion
Multiplex ligation dependent probe amplification (MLPA)	2	chromosome 15q11-13	Small deletion
Short tandem repeat linkage analysis (STR)	1	chromosome 15q11-13	Small deletion

syndrome. So far, detection methods for PWS include high resolution banding (HRB), fluorescence in situ hybridization (FISH), methylation-specific PCR (MSPCR), multiplex ligation dependent probe amplification (MLPA), short tandem repeat (STR) linkage analysis, microsatellite analysis, Southern blots, and so on (*7,8*). Shen *et al*. found abnormal methylation of the CpG islands in the *SNRPN* gene locus in a PWS patient using bisulphate sequencing (*7*). Che *et a*l. recently reported a PWS case using FISH, which showed a gene deletion in chromosome 15 q11.2 (*9*). Furthermore, a previous study showed that FISH and MSPCR when applied to 4 clinically suspected PWS patients and molecular pathogenesis of them had paternal micro-deletions of 15q11-q13 or maternal uniparental disomy of chromosome 15 (*10*). However, FISH cannot detect uniparental disomy or imprinting mutations. MSPCR is a sensitive, efficient, specific and convenient assay for detecting PWS. Recently, Gao *et al*. diagnosed a PWS patient with MSPCR and suggested that the clinician should diagnose suspected PWS patients with a methylation test and assure timely intervention (*11*). Previous studies showed that only MSPCR could detect deletion, uniparental disomy and imprinting defects. Therefore, 99% of PWS could be diagnosed by MSPCR (*12-14*). Compared to MSPCR, MLPA assays can be applied to clarify the pathogenesis and provide a scientific basis for clinical diagnosis (*15*). MLPA's high sensitivity and specificity for deletion detection is the same as the "gold standard", such as FISH analysis or Southern blot based methylation analysis. Moreover, a recent study showed that short tandem repeat (STR) linkage analysis can identify the molecular defect of PWS cases quickly and accurately (*16*). With more STR loci analyzed and their polymorphism information content obtained, this linkage analysis method could be used for a potential diagnosis in PWS cases. In addition, Li *et al*. established a linkage analysis method for Chinese patients. This method can detect both deletion and uniparental disomy, thus providing valuable information for genetic counseling and the opportunity to analyze the relationship between the PWS genotype and phenotype (*17*). In our study, we found that genome-wide copy number variation analysis is an effective and specific diagnostic method for PWS (Table 1).

With improved recognition and availability of testing methodologies, PWS is being diagnosed earlier, often in the first few months of life. Earlier diagnosis allowing for earlier access to developmental resources, recombinant human growth hormone therapy, and anticipatory guidance, has significantly improved the long-term health and developmental outcomes of children with PWS (*18*).

In conclusion, we found that genome-wide copy number variation analysis is an effective and specific diagnostic method, which will provide a guide for the clinical diagnosis and early treatment of PWS. Early diagnosis and comprehensive care of PWS patients have improved outcomes. However, areas where further research is needed include the etiology and management of PWS.

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Commentary

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Leiomyosarcoma: Principles of management

Juan Martin-Liberal*

The Royal Marsden Hospital, London, UK.

Summary **The term soft-tissue sarcomas (STS) embraces more than 50 different sub-types that are often associated with poor prognosis. Only a very limited number of agents are active against STS. Doxorubicin and ifosfamide are widely accepted as the most effective compounds. However, their low response rates and poor impact on the overall survival of the patients illustrate the need for new treatment options. Among them, leiomyosarcomas are one of the most frequently occurring subtypes. In spite of the relatively high incidence of leiomyosarcomas, the overall effectiveness of the currently available systemic treatments is still poor. The heterogeneity of its biological origin, clinical behavior and responsiveness to chemotherapy, together with the scarcity of successful clinical trials, makes the treatment of leiomyosarcoma especially challenging. In addition, the evidence-based treatment for leiomyosarcoma comes from trials in which, in the majority of cases, no distinctions have been made among the different STS sub-types. As a result, every therapeutic decision should be made on an individual basis in collaboration with the patient. The results of new specific histology-designed clinical trials should aid decision making in this complex field.**

Keywords: Leiomyosarcoma, sarcoma, targeted therapies, chemotherapy, hormone treatment

Soft-tissue sarcomas (STS) are a heterogeneous group of malignancies characterized by both their relatively low incidence and their poor prognosis. Classically, they have been treated as a single disease with rather disappointing results in the advanced setting. Thus, in spite of the wide range of systemic therapies available in oncology, only a very limited number of agents are active against sarcomas. Doxorubicin and ifosfamide are widely accepted as the most effective compounds. However, their low response rates (*1,2*) and poor impact on the overall survival of the patients (*3*) illustrate the need for new treatment options.

Leiomyosarcoma is one of the most frequent STS histologies with well-defined characteristics (*4*). It has been classically reported as the most frequent sarcoma sub-type together with liposarcoma (*5*). This high incidence might be due to the fact that, under the common label of leiomyosarcoma, there are a number of malignancies that differ in their biological behavior and, subsequent response to treatment. It is classically considered that leiomyosarcomas are tumors that originate from the smooth muscle cells, or precursor mesenchymal stem cells committed to this line of differentiation (*6*). As these cells are present practically in all organs, leiomyosarcomas can arise anywhere in the body. Indeed, their different behavior and sensitivity to treatment is often influenced by the site of origin. Although this observation is not exclusively confirmed in the literature (*7*), uterine leiomyosarcomas seem to be more sensitive to chemotherapy than those which arise in the vessels but vary among themselves in terms of grade and aggressiveness (*8*).

Within this broad spectrum of different origins and clinical features we can perceive distinct malignancies probably driven by different molecular alterations (*9*). However, the evidence-based treatment for leiomyosarcoma comes from trials in which, in the majority of cases, no distinctions have been made among the different sub-types.

Fortunately, the increasingly accepted change in the paradigm of the treatment of STS makes the future more promising. Thus, increasing knowledge of their molecular characterization has helped us in understanding that "STS" can no longer be considered

**Address correspondence to:*
Dr. Juan Martin-Liberal, The Royal Marsden Hospital, Fulham Road SW3 6JJ, London, UK.
E-mail: juan.martin@rmh.nhs.uk

as a single disease. For instance, there are sarcomas whose malignant behavior is clearly related to specific chromosomal translocations that lead to aberrant chimeric proteins. Others have complex karyotypes where the driver or drivers is unknown, whereas in other diseases, such as GIST, a specific gene mutation is responsible for driving the uncontrolled growth and resistance to death of the neoplastic cells (*10*). Together with this molecular heterogeneity, their intrinsic low incidence is an added difficulty. In order to enroll a sufficient number of patients to reach statistically significant results, the classical design of the trials allowed a broad spectrum of different histologies. Leiomyosarcomas have always been very well represented in these studies but the lack of stratification has led to results that are difficult to interpret. Some trials have even been reported as negative when the treatment assessed might have efficacy in certain subtypes. However, the clinical trials developed in the last few years have attempted to minimize these problems. Therefore, the increasing tendency is to focus on a specific sub-type or sub-types of sarcoma and also to use different statistical approaches. For instance, the EORTC in 2002 proposed new criteria of efficacy in sarcoma phase II trials in an attempt to reach achievable endpoints, with the emphasis on progression-free rates (*11*). The results of these new clinical trials began to be reported recently and, hopefully, more data will be available in the near future.

Probably for the same reasons as with cytotoxic drugs, the new targeted therapies have not achieved significant results so far. The lack of a specific target has been a handicap in the development of effective targeted drugs for the majority of solid tumors, including sarcomas. A family of targeted agents that has been widely assessed has been the multi-targeted tyrosine kinase inhibitors, perhaps principally inhibitors of VEGFR. However, few have achieved encouraging results as monotherapy. The only successful trial for advanced disease published to date (the PALETTE study, with pazopanib) did not demonstrate significant activity specifically in leiomyosarcoma patients and the overall results, although positive, are still limited (*12*). The strategy of combined treatment with targeted agents and classic cytotoxic drugs is an alternative approach, worthy of exploration. Most of the new targeted compounds recently developed in oncology produce an arrest or a slowdown in the growth of tumor cells but they may not cause cell death. Based on that premise, the rationale for combining these with cytotoxic drugs, that do effectively produce cell death, is very sensible. However, the main concern of this approach is toxicity and, unfortunately, this issue has already been proved to be potentially relevant in STS as was shown in a study by D'Adamo *et al* (*13*). New trials such as the one Gynecologic Oncology Group is currently conducting (with gemcitabine plus docetaxel plus bevacizumab in patients with advanced or recurrent uterine leiomyosarcoma) are necessary to determine whether this combined strategy is both feasible and safe.

Hormone therapy is also, conceptually, an attractive alternative for hormone-receptor positive leiomyosarcomas. Nevertheless, there are no randomized trials to date to help define the role of this treatment. Unlike endometrial stromal sarcomas, the only data available in leiomyosarcomas are just from small case series (*14-17*). The limited evidence that can be extracted from these suggests that hormone therapy might be a sensible strategy in the advanced setting for ER/PgR positive tumors with indolent growth. Otherwise, chemotherapy should be the treatment of choice.

With all these data, and considering the relative lack of evidence for the optimal treatment of leiomyosarcoma, every therapeutic decision should be made on an individual basis. For instance, adjuvant treatment may be considered in patients with a high-risk of recurrence, even though there are no randomized trial data that support it. The criteria indicative of increased likelihood of relapse for abdominal leiomyosarcomas (mostly uterine) such as tumor rupture during surgery or serosal breach are sufficient indicators of poor prognosis that adjuvant treatment may be justified. The choice of the appropriate regimen should take into consideration features like the special responsiveness that uterine leiomyosarcomas seem to have to gemcitabine and docetaxel. On the other hand, vascular leiomyosarcomas like the ones that arise from the inferior vena cava are generally considered to be particularly chemo-resistant so the real value of post-operative chemotherapy is unclear. The option of adjuvant hormone therapy might be sensible if the tumor is hormone-receptor positive, the risk of recurrence is high and the patient is not keen or is not fit enough to receive the standard chemotherapy. Also in the advanced setting, some special characteristics of leiomyosarcomas could determine the therapeutic strategy. These tumours have historically been shown to be not very sensitive to ifosfamide, so probably this drug should not be considered as the first line of treatment (*18*). As in the adjuvant setting, the special effectiveness of gemcitabine and docetaxel in gynaecological leiomyosarcomas makes it a valid option as frontline treatment instead of anthracyclines although results of direct comparison between the two regimens have not yet been reported.

In conclusion, individualized treatment must be the standard of care in a malignancy with such limited therapeutic options as leiomyosarcoma. This lack of very effective treatment makes it strongly advisable that patients should be enrolled in suitable clinical trials with new therapeutic strategies.

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Commentary

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Therapeutic strategies for Leber's hereditary optic neuropathy: A current update

Nuri Gueven, Dharmesh Faldu

School of Pharmacy, University of Tasmania, Hobart, Australia.

Summary **Leber's hereditary optic neuropathy (LHON) is a rare mitochondrial retinopathy, caused by mutations in subunits of complex I of the respiratory chain, which leads to elevated levels of oxidative stress and an insufficient energy supply. This molecular pathology is thought to be responsible for the dysfunction and eventual apoptotic loss of retinal ganglion cells in the eye, which ultimately results in blindness. Many strategies, ranging from neuroprotectants, antioxidants, anti-apoptotic- and anti-inflammatory compounds have been tested with mixed results. Currently, the most promising compounds are short-chain quinones that have been shown to protect the vision of LHON patients during the early stages of the disease. This commentary gives a brief overview on the current status of tested therapeutics and also addresses future developments such as the use of gene therapy that hopefully will provide safe and efficient therapy options for all LHON patients.**

Keywords: Leber's Hereditary Optic Neuropathy, mitochondrial disease, quinones, therapy, clinical trial, antioxidants, neuroprotectants

1. Introduction

Leber's hereditary optic neuropathy (LHON) is a retinal neurodegenerative disorder, characterized by acute or subacute vision loss in one eye, generally followed by loss of visual acuity in the second eye within 2-4 months (*1,2*). Loss of vision in LHON patients is associated with dense central or centrocecal scotoma and impaired color vision. LHON predominantly affects young adult males of all ethnic groups, with a peak of onset in young adulthood. While in most cases vision loss is permanent, a minority of patients show spontaneous recovery of visual acuity by an unknown mechanism (*1,2*). LHON is regarded as one of the most prevalent mitochondrial diseases with an incidence between 1:30,000 and 1:50,000 (*2,3*). However, it can be assumed that LHON is still significantly underdiagnosed as optic atrophy of unknown origin. More than 100 years after the initial description of the disease, the first causative point mutation in the mitochondrial DNA (mtDNA) was identified (*4*) and at present more than 18 mtDNA alterations have been associated with LHON (*http://omim.org/entry/535000*). However, three so called primary mtDNA mutations account for about 95% of all LHON cases, 11778G>A (*ND4* subunit), 14484T>C (ND6 subunit), 3460G>A (*ND1* subunit), all of which lead to a dysfunction of complex I of the mitochondrial electron transport chain (*2*). As a result of this defect, decreased ATP synthesis and elevated levels of oxidative stress have been described (*5-7*), which are thought to impair the function and ultimately lead to apoptotic cell death of retinal ganglion cells (RGC) (*6-9*). Despite progress towards a better understanding of pathogenesis of LHON, currently no treatment options are available to patients and only a few years ago LOHN was regarded as untreatable. Furthermore, due to the spontaneous recovery potential seen in some LHON patients, any reports of treatment efficacy from small uncontrolled trials must be considered with extreme caution (Table 1). However, recent clinical data with redox-active electron carriers have demonstrated that protection and even recovery of vision is a realistic prospect in LHON.

**Address correspondence to:*
Dr. Nuri Gueven, School of Pharmacy, University of Tasmania, Private Bag 26, Hobart, TAS 7001, Australia.
E-mail: nguven@utas.edu.au

2. Neuroprotectants, anti-inflammatories and anti-apoptotic compounds

Given that the pathology of LHON seems to be largely

Table 1. Clinical results of different therapy approaches for the treatment of LHON

#	Reference /Sponsor*	Treatment	Dose	Type of study	Duration	No. of Patients	Primary Measure	Outcome	Clinical trial number	status
1	Newman *et al*. 2005 (*15*)	Brimonidine	0.15% 4 × daily	open-label, non-randomized prospective	2 years	9	Visual acuity	Brimonidine was unsuccessful in preventing second eye involvement. Trial closed prematurely because of low enrolment rate, and all patients failed the criteria for effectiveness during follow-up	n/a	finalized
2	Medical College of Wisconsin 2011	Near-infrared-light-emitting diode	50 mW/cm^2 2 × daily	non-randomized	12 months	4	Pre- and post-treatment electroretinogram	Unable to record primary measure as patients were unable to focus on target, no study results posted	NCT01389817	terminated
3	Buhmann *et al.* 2002 (*16*)	Mitoxantrone	12 mg/m^2 every 3-4 months	case study	48 months	1	Visual acuity	Recovery of visual function in patient with 11778G>A mutation 12 month after onset of vision loss	n/a	finalized
4	Huang *et al*. 2002 (*18*)	CoQ10	90-200 mg/day	case study	12 months	1	Visual acuity	Recovery of visual function in patient with 11778G>A mutation	n/a	finalized
5	Carelli *et al.* 2011 (*24*)	Idebenone	270-675 mg/day	non-randomized, retrospective	variable	103	Visual acuity	Increased recovery of vision in the treated group	n/a	finalized
6	Klopstock *et al*. 2011, 2013 (*23,25*)	Idebenone	900 mg/day	placebo-controlled, randomized, double-blind	6 months	85	Visual acuity	Protection against loss of visual acuity, improvement of visual acuity, treatment effect persisted even 30 months after termination of treatment	NCT00747487 NCT01421381	finalized
7	Sadun *et al*. 2012 (*28*)	EPI-743	300-1200 mg/day	non-randomized, open label	204-557 days	5	Anatomic and visual indices	4 out of 5 treated patients showed improvement of visual function	n/a	finalized
8	Mahidol University 2007	Curcumin	2 × 250 mg/day	placebo-controlled, randomized, double-blind	12 months	70	Visual acuity, visual field, electrophysiology	No study results released	NCT00528151	finalized
9	Quark Pharmaceuticals 2010	QPI-1007	variable doses	open-label, dose escalation, safety, tolerability study	12 months	48	Safety,dose-limiting toxicities, pharmacokinetics	No study results released	NCT01064505	finalized
10	Huazhong University of Science and Technology 2010	rAAV2-ND4, gene therapy	single injection	Safety and efficacy study	6 months	6	Visual acuity	Final data collection due by end of 2013	NCT01267422	recruiting

*Sponsor is listed for studies with no published results.

restricted to a degeneration of RGC cells, it appears obvious that any protection of RGC neurons against cell death should at least in theory alleviate the symptoms associated with LHON. Many compounds with RGC-neuroprotective properties such as memantine (*10*), valproic acid (*11*) and SIRT-1 activators (*12*) have been identified in other models and could also be tested in pre-clinical models of LHON and/or in clinical trials. Some promising pre-clinical results have been reported with compounds such as the antibiotic drug minocycline (*13*). However, unless their efficacy is confirmed in tightly controlled clinical trials, their potential for the treatment of LHON patients remains unclear. Many putatively protective compounds such as steroids, hydroxycobalamin and cyanide antagonists have been tested to treat or prevent the acute phase of vision loss, but without success (*14*). In one of the few clinical trials, the anti-apoptotic compound brimonidine, approved to treat ocular hypertension and open-angle glaucoma, was tested in an open-label, non-randomized prospective study (*15*). Although the LHON patients in this trial were in the early stages of the disease with discordant vision and had therefore the highest chance of recovery, brimonidine was unsuccessful in preventing second eye involvement. Curcumin, a compound that has also been associated with many protective activities, was tested in a placebo-controlled trial that started in 2007 (NCT00528151). However, the absence of published results and the lack of a follow up study at present suggest that probably no positive effects were obtained with curcumin. One interesting observation is derived from a patient suffering from the rare LHON-multiple sclerosis (MS)-like disease. Treatment of this single patient with the commonly used anti-MS drug mitoxantrone resulted in a time delayed visual recovery 12 months after acute onset of rapid sequential bilateral subtotal visual loss, which led the authors to suggest an immunological involvement in the pathology of this disorder (*16*). However, the structural similarity of mitoxantrone to naphthazarin could also suggest a direct neuroprotective activity (*17*). An entirely different approach is the use of gene silencing to down regulate the expression of apoptosis-inducing genes such as in the case of the experimental compound QPI-1007, which targets the expression of the pro-apoptotic enzyme caspase 2 (NCT01064505). Phase 1 safety and tolerability have been assessed in a dose escalation trial in healthy subjects and it remains to be seen how effective this approach will be when used in optic atrophy patients.

3. Antioxidants and Electron Carriers

Given the good evidence for elevated levels of oxidative stress in LHON, antioxidant treatment has been proposed or tested repeatedly. While pre-clinical models showed sufficient efficacy to take some compounds into the clinic, compounds that act as radical scavengers only, have so far failed to show convincing clinical evidence of usefulness. On the other hand, promising clinical results have recently been described with molecules that can reduce oxidative stress levels and simultaneously act as electron carriers to modulate mitochondrial electron flow. The most well-known member of this group is the endogenous coenzyme Q10 (CoQ10), which is utilized in many mitochondrial disorders. Although, a beneficial effect of CoQ10 in a single patient harboring the 11778G>A mutation was reported (*18*), results with CoQ10 in controlled trials in other mitochondrial indications have so far not been convincing (*19*), most likely due to its poor tissue bioavailability as a consequence of its very high lipophilicity. In contrast, idebenone, a short chain benzoquinone has shown some encouraging effects (*20*). Due to its balanced lipophilicity (logD = 3.9), it not only acts as a potent catalytic antioxidant but can shuttle between the cytoplasm and the mitochondria to transfer electrons into the mitochondrial electron transport chain under conditions where complex I is defective (*21,22*). This mechanism is dependent on the cellular levels and activity of NAD(P)H oxidoreductase (NQO1) and although there is no direct evidence that idebenone acts *via* an NQO1-dependent mechanism in LHON patients, biochemical data demonstrated a normalization of mitochondrial function associated, for example, with reduced lactate production (*21*). A number of earlier case studies and trials, which suggested that idebenone could have a therapeutic effect in LHON patients (*20*), provided the rationale for the first randomized, placebo-controlled study in LHON. This randomized placebo-controlled clinical trial (RHODOS, Rescue of Hereditary Optic Disease Outpatient Study; NCT01421381) included eighty five LHON patients carrying one of the three primary mtDNA mutations and were treated with 900 mg of idebenone per day for 24 weeks (*23*). Although patients receiving idebenone improved on average by six letters while subjects receiving placebo improved by three letters, this trial did not reach is pre-specified endpoint of "best recovery in visual acuity in either eye" measured by change in logMAR between baseline and week 24. However, when analyzing the change of all subjects' eyes separately to increase the power of the study (pre-specified secondary endpoint), the visual acuity of eyes of patients receiving idebenone significantly improved compared to those receiving placebo (p = 0.026). Furthermore, 28% of patients receiving idebenone and unable to read the eye-chart at baseline recovered sufficient visual acuity to read at least five letters on the eye chart compared to 0% of patients in the placebo group (*23*). The results from the RHODOS trial, together with similar data from a non-randomized, retrospective trial of idebenone in LHON that involved 103 patients (*24*) support a protective and

restorative activity of idebenone in patients, particularly those with recent disease onset. Interestingly, when the patients of the RHODOS trial were followed up 30 months after treatment had been terminated, it became clear that the protective idebenone effect still persisted (*25*).

A second quinone compound, which unlike idebenone belongs to the Vitamin E family of compounds is α-tocotrienol-quinone (EPI-743), was tested in small trials in several mitochondrial indications such as Leigh syndrome (*26,27*). At present, EPI-743 was evaluated only in a single open-label clinical trial involving five LHON patients with acute vision loss, where visual function improved in four out of five patients based on visual acuity or visual field (*28*). Similar to idebenone, EPI-743 is reduced by the enzyme NADPH quinone reductase (NQO1) however at a rate of less than 30% compared to idebenone (*29*). Due to its higher lipophilicity, it does not participate in the cytoplasmic-mitochondrial electron shuttling reported for idebenone (*29*). Based on the available literature, the most likely mode of action of this redox-active compound could be a strong antioxidative effect (*30*). Even though the clinical results with EPI-743 appear promising and raise hope in many LHON patients, they still have to be verified by tightly controlled studies with sufficient patient numbers.

It has to be noted here that a strict classification of compounds as antioxidants, neuroprotectants or electron carriers is realistically not possible, since many of the described molecules could potentially display several activities at once. This highlights the problem that even when compounds show activity in protecting visual acuity in LHON patients, we cannot necessarily deduce by what mechanism, which hinders the development of compounds with increased potency. Moreover, this uncertainty also makes claims of superiority of one compound over another based on pre-clinical data largely unfounded until a clear improved activity has been demonstrated in controlled, comparative clinical trials.

4. Gene therapy

In addition to pharmacological approaches, there is significant activity to directly correct the inherited genetic defect by expressing the functional mitochondrial protein in the retina (*31-33*). The application of gene-therapy in LHON is mainly based on the nuclear, allotopic expression of mtDNA-encoded genes, where the wild type version of the mutant mitochondrial complex subunit is delivered into the RGC *via* adeno-associated virus (AAV) (*31*). The underlying idea of this approach is that proteins that are normally expressed in the mitochondria can be produced in the cytoplasm and then imported into the mitochondria using specific mitochondrial targeting sequences. Once in the mitochondria, it is assumed that they can be correctly incorporated into the mitochondrial enzyme super-complexes to replace the defective subunits, thereby restoring normal electron flow and energy production. Although a significant critique of this approach has been voiced (*32,33*), successful results were reported using pre-clinical *in vitro* and *in vivo* models of LHON (*31,34*). Currently several clinical trials are in preparation or ongoing that universally aim to treat LHON patients by expressing the wild-type form of the *ND4* gene using AAV vectors (NCT01267422) (*35,36*). It has to be noted that due to its nature this approach will not be useful for all those patients that harbor mutations in different subunits than ND4. In this context it is therefore interesting to note that a second gene-therapy approach could be independent of the underlying mutation. This so far only pre-clinical work is based on the expression of a yeast-derived NADH-oxidase called Ndi. Encouragingly, the mitochondrial expression of this enzyme in a mouse model of LHON protected against RGC loss and preserved visual function (*37*). It will be exciting to see the first results of clinical gene-therapy trials in this field. However, these studies not only have to demonstrate sufficient efficacy with regards to a protection and restoration of visual acuity in LHON patients but also need to display a tolerable safety profile to become a viable treatment option either as independent treatment modalities or in combination with pharmacological strategies.

5. Conclusion

At present, effective and available treatment options for all LHON patients are still lacking. However, based on the recent encouraging results with quinone compounds in some patients in the early stages of the disease, LHON can no longer be seen as an untreatable disorder. Current evidence also suggests that the development of LHON-specific gene-therapy approaches could add to the therapeutic repertoire in the future. Over the next few years, a detailed understanding of the molecular pathology combined with the use of pharmacological therapies and potential genetic treatment options will therefore likely lead to significant therapeutic improvements for all LHON patients.

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Letter

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Can Alzheimer's disease be prevented?

Frank Murray*

New York, USA.

Summary **Alzheimer's disease (AD) is the most common form of dementia. Survival time for an AD patient is generally 4 to 6 years after diagnosis, however, survival time can be as long as 20 years from the detection of initial symptoms, which can surface in the 30s, 40s, and beyond. This window of opportunity suggests that many people can prolong their life with life-changing choices related to diet, exercise, nutritional supplements, and nutraceuticals. This was emphasized in many recent studies and was described in detail in the book "Minimizing the Risk of Alzheimer's Disease" published in the USA in 2012.**

Keywords: Alzheimer, risk factor, smoking, total dietary fat, regular exercise

Due to the aging population globally, there are currently an estimated 35.6 million people with dementia, according to the World Health Organization, Geneva, Switzerland. That number is expected to almost double every 20 years, reaching 65.7 million in 2030, and 115.4 million in 2050 (*1*). Alzheimer's disease (AD) is the most common form of dementia, with others being multi-infarct dementia (vascular dementia/post-stroke), Creutzfeldt-Jacob disease, Pick's disease, Parkinson's disease, Lewy body disease, Huntington's disease, and others, reported by the Alzheimer's Association, Chicago, Illinois (*2*).

The prevalence of AD in Brazil, China, Cuba, Egypt, India, Nigeria, Republic of Korea, and Sri Lanka was addressed in a study, and it ranged from 1.3% in India to 8.0% in Korea. Survival time for an AD patient is generally 4 to 6 years after diagnosis, however, survival time can be as long as 20 years from the detection of initial symptoms, which can surface in the 30s, 40s, and beyond. This window of opportunity suggests that many people can prolong their life with life-changing choices related to diet, exercise, nutritional supplements, and nutraceuticals, and was emphasized by the book "Minimizing the Risk of Alzheimer's Disease" (Algora Publishers, New York, USA, 2012).

In the September 22, 2013 issue of the Journal of Alzheimer's Disease, Hiroko H. Dodge, Ph.D., assistant professor of Neurology at Oregon Health and Sciences University in Portland, the lead researcher, reported dramatic increases in AD in Japan and other countries which are linked to changes in national diets (*3*). In Japan, for those who are over 65 years of age, AD rose from 1% in l985 to 7% in 2008, Dodge said. She has been conducting a cohort study on Okinawa in collaboration with a research team from the Okinawa Centenarian Study. The prevalence of vascular dementia was almost constant at 4% to 5% during the previously named period. In data she quoted from the Food and Agricultural Organization of the United Nations, the largest changes in Japan between 1961 and 1965 are, as follows: *i*) alcohol, from 29.6 kg/person/year to 57.4; *ii*) animal fat, from 5 kg/person/year to 35; *iii*) meat, from 7.6 kg/person/year to 33.7; *iv*) energy from animal products, from 249 kcal/person/day to 580; and *v*) rice, 113 kg/person/year to 89. Dr. Dodge added that the data linked animal products and meat to AD because of the iron in meat, which increases oxidative stress; arachidonic acid in meat, which increases inflammation in the brain; and cholesterol from animal products. She said that, since dietary factors haven't changed appreciably since 1985, perhaps AD prevalence rates has peaked, and will not increase in the future. "It is important to prevent or delay the onset of AD", she continued. "If we pay attention to what we eat, and do at least moderate exercise, we can significantly reduce the societal burden of dementia in the future".

In the book "Minimizing the Risk of Alzheimer's Disease", it was reported that over half of the AD cases in the world could be prevented if we eliminated those risk factors that we have control over, such as

**Address correspondence to:*
Mr. Frank Murray, 410 W. 24th St., 8I, New York 10011, USA.
E-mail: letslivefm@aol.com

depression, obesity, and smoking, either with lifestyle changes or treatment of the underling conditions (*4*). That was the conclusion of researchers at the Alzheimer's Association International Conference, held July 19, 2011 in Paris, France. In addition, a 25% reduction in 7 common risk factors could prevent up to 3 million AD cases around the world. Some of the risk factors have a greater impact on AD risk than others, according to Deborah Barnes, M.D., the University of California at San Francisco. Worldwide, she added, 19% of the AD cases can be linked to low education; 14% to smoking; 13% to physical inactivity; 10% to depression; 5% to hypertension; 2.4% to diabetes; and 2% to obesity.

It is not surprising that smoking is high on the "don't do" list. Compared with non-smokers, those who smoked 2 packs of cigarettes a day had more than a 15% risk of AD, and a 172% increased risk of vascular dementia, during the 23 years of follow-up in their study, according to a research team at the Kaiser-Permanente Division of Research in Oakland, California (*5*). Tobacco smoke contains over 4,000 chemicals, many of which are free-radicals, which are highly reactive compounds that damage cells and initiate a cascade of health problems (*6*). Antioxidant nutrients, including vitamin A, beta-carotene (pro-vitamin A), vitamin C, and vitamin E, help to neutralize these damaging reactions caused by tobacco smoke, but, unfortunately, antioxidant defenses are overwhelmed by the amount of free-radicals in tobacco.

Total dietary fat is a high risk factor for AD, while high intakes of monounsaturated fatty acids, found in the Mediterranean diet, have been shown to reduce cognitive decline, according to W. B. Grant, M.D. (*7*). Fish reduces the risk of developing AD, and linolenic acid, an omega-3 fatty acid, also found in nuts and seeds (flaxseed), has been inversely associated with AD, he said. Omega-6 fatty acids are found in vegetables. A sensible diet later in life appears to be more important than a diet earlier in life, Grant added. As an example, a proper diet 4 years prior to the onset of AD showed the best results. Also, a diet rich in cereals and grains is strongly inversely related to AD. In addition, the genetic predisposition to AD through apoprotein-E (ApoE) and diet are both important in the etiology of the disease. Grant added that vitamin E supplements have been shown to reduce the risk of AD, since this fat-soluble vitamin protects lipids/fats from nitric oxide-initiated peroxidative damage. An excess of nitric oxide, which is a free-radical, is toxic to brain cells. A colorless gas, NO is thought to affect immune reactions and memory. Homocysteine is normally a benign amino acid, but it can build to toxic levels and cause serious health problems. Over 20 case-controled and cross-sectional studies, involving 15,000 patients, support the role of elevated homocysteine levels and vascular disease, reported Ramon Diaz-Arrestia, M.D. (*8*). In another study, it was found that the upper one-third of serum homocysteine distribution had at least a 3-fold increased risk of developing AD. There was also an inverse relationship between blood levels of folic acid, the B vitamin, and vitamin B12, and the risk of AD.

The Mediterranean diet - high in vegetables, legumes, fruit, nuts, cereals, fish, olive oil, and low in saturated fats - has been linked to a lower risk of AD, according to James M. Ellison, M.D., of the Harvard Medical School in Boston, Massachusetts (*9*). White rice is a leading carbohydrate in many national diets, however, in a study involving 39,765 men and 157,463 women, who were enrolled in the Professional Follow-Up Study and the Nurses' Health Study I and II, a research team at the Harvard School of Public Health in Boston, reported that substituting brown rice for white rice may reduce the risk of Type 2 diabetes (*10*). Five or more servings of white rice per week brought a 17% increase in Type 2 diabetes, when compared with less than l serving per month. Most carbohydrate intake should come from whole grains rather than refined grains to prevent diabetes, they said. A study at Hanyang University in Seoul, Korea, reported that the consumption of 4 to 6 servings of vegetables daily brought a 32% reduced risk of stroke. Six servings daily brought a 69% reduced risk of that disease, after adjusting for potential cofactors (*11*).

Regular exercise during midlife may reduce the risk of AD and other dementias later in life, according to a research team in Sweden and Finland. The study involved 1,449 volunteers - ranging in age from 65 to 79 (*12*). Those who exercised at least twice a week for 20 to 30 minutes during midlife were at least 50% less likely to develop AD and other dementias, compared to those who were more sedentary, even after adjustments for vascular disorders, smoking, ApoE genotype, and other factors.

The book "Minimizing the Risk of Alzheimer's Disease" provides additional data on the importance of diet, exercise, nutraceuticals, drugs (the good, the bad, the ugly), the dangers of smoking, the complicated aspects of AD and how they are being addressed, how to prevent depression, how to get some sleep, the dangers of toxic metals, and more.

(*Frank Murray, a former editor of 3 prominent nutrition magazines, the author/coauthor of 54 books on health and nutrition published in the U.S., India, and China. He is also a member of the New York Academy of Sciences and lives in New York.*)

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Original Articles should be well-documented, novel, and significant to the field as a whole. An Original Article should be arranged into the following sections: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, and References. Original articles should not exceed 5,000 words in length (excluding references) and should be limited to a maximum of 50 references. Articles may contain a maximum of 10 figures and/or tables.

Brief Reports definitively documenting either experimental results or informative clinical observations will be considered for publication in this category. Brief Reports are not intended for publication of incomplete or preliminary findings. Brief Reports should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 4 figures and/or tables and 30 references. A Brief Report contains the same sections as an Original Article, but the Results and Discussion sections should be combined.

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Pearl City Koishikawa 603
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